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Quantitative Detection of *Escherichia coli* O157 in Surface Waters by Using Immunomagnetic Electrochemiluminescence

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A protocol for the quantitative detection of *Escherichia coli* O157 in raw and concentrated surface waters using immunomagnetic electrochemiluminescence (IM-ECL) was developed and optimized. Three antibody sandwich formats were tested: commercial anti-O157:H7 IM beads, IM beads made in-house with a polyclonal anti-O157:H7 immunoglobulin G (IgG), or IM beads made in-house with a monoclonal anti-O157:H7 IgG coupled with a polyclonal anti-O157:H7 IgG to which an electrochemiluminescent label (TAG) was attached. The monoclonal IM bead-polyclonal TAG format was chosen for optimization because it gave lower background levels and linear regression slopes of ca. 1.0, indicative of a constant ECL signal per cell. The dynamic range was ca. 10^1 to 10^5 cells ml^{-1} in phosphate-buffered saline and in raw water samples. The monoclonal IM beads selectively captured *E. coli* O157 cells in the presence of ca. 10^8 cells of a non-O157 strain of *E. coli* ml^{-1}. Background ECL signals from concentrated (100-fold) water samples were substantially higher and more variable than raw water samples. The background signal was partially eliminated by the addition of polyvinylpolypyrrolidone. Successive cell capture incubations, termed sequential bead capture (SBC), were optimized for establishing baseline ECL values for individual water samples. The linear dynamic range with SBC was ca. 10^2 to 10^5 *E. coli* O157 cells ml of concentrated $water^{-1}$. To validate the protocol, 10-liter surface water samples were spiked with ca. 5,000 *E. coli* O157 (Odwalla) cells and concentrated by vortex filtration, and 1- or 3-ml aliquots were analyzed by IM-ECL. Differential ECL signals (SBC) from 1- and 3-ml samples were statistically significant and were generally consistent with standard curves for these cell concentrations. Enrichments were conducted with aliquots of spiked raw water and concentrated water using EC broth and minimal lactose broth (MLB). All tubes with concentrated water became turbid and gave a positive ECL response for *E. coli* O157 (>10,000 ECL units); MLB gave a somewhat higher detection rate with spiked raw water. The potential sensitivity of the IM-ECL assay is ca. 25 *E. coli* O157 cells ml of raw $water^{-1}$, 25 cells 100 ml of 100-fold concentrated $water^{-1}$, or 1 to 2 viable cells $liter^{-1}$ with concentration and enrichment. The IM-ECL assay appears suitable for routine analysis and screening of water samples.

Enterohemorrhagic *Escherichia coli* (EHEC) has emerged as a serious food-borne and waterborne pathogen. There are an estimated 73,000 cases of *E. coli* O157 infections per year in the United States, of which approximately 62,000 are food-borne and 11,000 are waterborne (14). *E. coli* O157 was first reported in the United States in 1982, when it was associated with a multistate outbreak of hemorrhagic colitis (20). EHEC outbreaks (including O157 and O111 strains) have since been reported in Europe and Australia (2). In 1986, *E. coli* O157 was recovered from healthy dairy cows, suggesting that dairy and beef herds could serve as a reservoir (13). Subsequent studies have confirmed that *E. coli* O157 and other EHEC strains are commonly found in beef and dairy cattle (6, 19, 21, 24) as well as animals associated with farm environments: birds, flies, rodents, and companion animals (8). Recent studies suggest that deer may also be a source of *E. coli* O157 (11, 18).

Although the predominant mode of transmission to humans is contaminated meat or meat products, infection via contaminated water has also been documented. For example, Ackman et al. (1) reported an outbreak of *E. coli* O157 (six confirmed and six probable cases) among swimmers in a freshwater lake in New York State. The lake was closed by the county department of health 8 days after the presumptive exposure, and extensive water testing was conducted. However, no water samples exceeded New York standards (70 fecal coliform CFU ml^{-1}), and *E. coli* O157 was not detected in lake water samples, presumably due to die-off and/or dilution.

This case illustrates the difficulty associated with the detection and enumeration of *E. coli* O157 cells in surface waters. Cultural methods are laborious and expensive. For example, the current USDA-Food Safety and Inspection Service method entails four enrichment and culturing steps for preliminary identification and 10 biochemical tests for confirmation of *E. coli* O157 (5). These require a minimum of 3 days to perform. The use of sorbitol MacConkey agar has been proposed as a presumptive indicator of *E. coli* O157 because most O157 strains are sorbitol negative while generic *E. coli* strains typically are sorbitol positive (10). However, many genera of enteric bacteria contain sorbitol-negative species or strains. Consequently, multiple sorbitol-negative colonies must be screened via genetic, biochemical, or serological methods to accurately quantify *E. coli* O157 populations. In addition, standard enrichment methods for detection and enumeration of fecal *E. coli* cells via most-probable-number techniques may fail to detect *E. coli* O157. Ferenc et al. (7) reported that many O157 strains did not grow at 44.5°C in EC broth (ECB) when their initial populations densities were <100 CFU ml^{-1}.

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Kusunoki et al. (12) evaluated the growth of *E. coli* O157 at 37 and 42°C in six different media. *E. coli* O157 grew more rapidly at 37°C in all media, with the exception of Trypto-soya broth. Therefore, incubation at 37°C is advisable; however, because many bacteria have growth optima at or near 37°C, *E. coli* O157 may be out-competed by other bacterial strains when present at lower population densities.

Recently, a variety of immunological methods have been developed for the detection and enumeration of *E. coli* O157 cells. The common denominator among all methods is the use of monoclonal or polyclonal anti-*E. coli* O157 antibodies to selectively capture, or capture and label (sandwich assay), *E. coli* O157 cells. For example, several investigators have developed protocols for the enumeration of viable *E. coli* O157 cells using immunomagnetic (IM) bead separation (IMS) techniques in conjunction with plating (3, 6). Several sandwich assays have also been described that use a variety of methods for capture, labeling, or detection. Pyle et al. (17) described a method utilizing IMS and immunofluorescent antibody (IFA) techniques for capture and labeling, respectively, followed by enumeration via solid-phase laser cytometry. Kusunoki et al. (12) described a similar method utilizing immunolatex beads in conjunction with IFA, followed by flow cytometry. Park and Durst (15) reported a sandwich assay in which *E. coli* O157 cells were immobilized by anti-*E. coli* O157 antibodies bound to nitrocellulose and detected using immunoliposomes containing a marker dye. DeMarco et al. (4) developed a sandwich assay using anti-*E. coli* O157 antibodies bound to silica fibers for capture, IFA for labeling, and a fiber-optic sensor for quantitation. Finally, Yu and Bruno (23) described a method utilizing IMS in conjunction with electrochemiluminescence (ECL) for labeling and detection. Although technically identical to the method reported here, they optimized their protocol only for the qualitative detection of *E. coli* O157 and *Salmonella enterica* serovar Typhimurium in various foods and water samples. All of the above methods have been demonstrated to be suitable for the detection of *E. coli* O157 in extraction buffers or enrichment media derived from fecal, food, or water samples. However, none have been shown to be applicable to the detection of *E. coli* O157 in raw or concentrated surface water samples containing variable levels of sediments, organic particulates, and unidentified microflora.

We report here on the development and optimization of an IM-ECL protocol for the quantitative detection of *E. coli* O157 in raw and concentrated surface water samples. The dynamic range of the assay in raw surface water samples is ca. 10^1 to 10^5 *E. coli* O157 cells ml^{-1}. In conjunction with vortex filtration concentration (100-fold), the detection limit is ca. 25 *E. coli* O157 cells 100 ml^{-1}. When concentration and enrichment are combined, the potential detection limit is 1 to 2 viable cells $liter^{-1}$.

MATERIALS AND METHODS

Bacteria, growth conditions, and reagents. EC broth (ECB) and membrane fecal coliform (mFC) media were purchased from Difco Laboratories (Detroit, Mich.). Minimal lactose broth (MLB) consisted of the basal salts medium of Hylemon and Phibbs (9) (50 mM potassium phosphate, 15 mM ammonium, and trace nutrients) modified by the addition of 8.5 g of NaCl, 1.5 g of Bacto Bile Salts (Difco), and either 3.6 g of lactose (10 mM) or 0.36 g of lactose (1 mM) $liter^{-1}$. Phosphate-buffered saline (PBS-2) consisted of 150 mM potassium phosphate buffer (pH 7.2), 150 mM NaCl, and 0.1% azide, while PBS-1 consisted of potassium phosphate plus 150 mM NaCl at pH 7.8. Diluent consisted of PBS-2 amended with 4% (wt/vol) bovine serum albumin (BSA) and 1% (vol/vol) thesit (polyoxyethylene 9 lauryl ether).

E. coli O157 strains Odwalla and B6914 were obtained from Pina Fratamico (USDA-Agricultural Research Service, Eastern Regional Research Laboratory, Wyndmoor, Pa.). Strain ATCC 35150 was purchased from the American Type Culture Collection (Manassas, Va.). A sorbitol-negative non-O157 *E. coli* strain, strain 794, was isolated from a lactating dairy cow at the Beltsville Agricultural Research Center. Bacteria were cultured in Lennox broth for 18 h at 37°C with shaking. Bacteria were harvested from the cultures by centrifugation at 6,000 × *g* for 15 min at 4°C and washed by suspension in cold 50 mM potassium phosphate buffer (pH 7) containing 0.85% sodium chloride (PBS). Sodium azide (0.1%) was added to the suspended cells as a preservative, and they were stored at 4°C. Cell numbers were determined in a hemocytometer using phase-contrast microscopy (magnification, ×400; average of six determinations). Concentrations of bacterial stock solutions were 2.0×10^9, 2.4×10^9, and 3.2×10^9 cells ml^{-1} for B6914, Odwalla, and 35150 strains, respectively. To assess the effect of growth media on ECL signal, *E. coli* O157 (Odwalla) cells were grown in 1 mM MLB with or without bile salts to ca. 2×10^8 cells ml^{-1}; precise numbers were determined using a hemocytometer (six determinations). Cultures were serially diluted with PBS-2, and the 10^2 to 10^4 dilutions were analyzed by IM-ECL (data are means of three cultures).

Glycerol, Triton X-100, polyethylene glycol 1000 (PEG 1000), PEG 8000, BSA, and polyvinylpolypyrrolidone (PVPP) were purchased from Sigma (St. Louis, Mo.) while hexa meta-phosphate and EDTA were purchased from Fisher (Pittsburgh, Pa.).

Surface water collection and concentration. Ten-liter water samples were collected on 27 October 1999 from Little Cove Creek and Licking Creek, and on 7 July 2000 from Little Cove Creek, located in the Conococheague-Opequon watershed (U.S. Geological Survey no. 02070004) in south-central Pennsylvania. The creeks drain a watershed containing a combination of forest and pasture (dairy). The creeks flow into the Potomac River near Hancock, Md.

Ten liters of water was concentrated 100-fold using a Benchmark GX vortex filtration unit, manufactured by Osmotics (Minnetonka, Minn.). Raw water was pumped through the unit using a peristaltic pump (flow rate of 150 ml min^{-1}) followed by an additional liter of distilled water to rinse the tubing. An MX 500 (50-nm-pore-size) 200-cm^2 cylindrical filter cartridge spinning at 2,000 rpm was used. The unit was drained (ca. 40 to 50 ml), a liter of distilled water was pumped through the unit to wash the filter, and the unit was drained again (ca. 40 to 50 ml). The concentrated water and wash water were combined, and the total volume was adjusted to 100 ml with distilled water. The filtration unit was cleaned with 1 liter of 0.1 N NaOH between runs. Concentrated samples were preserved either by adding 10× PBS-2 (10%, vol/vol) or by adding the equivalent amounts of phosphate, salt, and azide dry reagents to give a 1× PBS-2 concentration. Based on preliminary data which indicated that removal of particulates enhanced cell capture, prior to analysis, 13-ml aliquots (in 15-ml tubes) were centrifuged at 100 × *g* for 15 min to sediment soil particulates and the top 10 ml was transferred to a clean tube.

Turbidity (in nephelometric turbidity units) was determined before and after concentration using a model 965-10A Turbidimeter manufactured by Orbeco Analytical Systems, Inc. (Farmingdale, N.Y.). Selected concentrated water samples were filtered using cellulose acetate syringe filters with a pore size of 0.2 μm (Gelman Sciences, Ann Arbor, Mich.).

Water spiking and enrichment. Water spiking experiments were conducted with *E. coli* O157 Odwalla cells grown to stationary phase in 1 mM MLB at 37°C. Cultures were diluted ca. 10,000-fold in PBS, and 1 ml was added to 10 liters of Little Cove Creek water samples (collected 7 July 2000). Just prior to concentration, total coliforms counts were determined by filtering 100 ml of water sample and transferring the filters to mFC media at 37°C. After spiking and concentration, *E. coli* O157 CFU were determined on MacConkey or Lennox agar with an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda, Md.). CFU were counted manually or with a Protocol plate reader (Synoptics, Cambridge, United Kingdom).

Enrichment experiments were conducted by mixing 4 ml of raw or concentrated water (three replicates) with 4 ml of 2× ECB or 2× MLB and incubating the mixtures statically at 37°C until turbid. When turbid, 1 ml of sample was transferred to a microcentrifuge tube and centrifuged for 10 min at high speed (ca. 13,000 × *g*), the supernatant was discarded, and the pellet was suspended in 0.5 ml of PBS-2. Samples were diluted 10^2-, 10^4-, and 10^6-fold with PBS-2, and 0.5 ml was analyzed via the IM-ECL protocol as subsequently described.

IM bead preparation. Commercial *E. coli* O157 beads (Dynabeads) and 2.8-μm-diameter streptavidin beads (Dynabeads M-280) were manufactured by Dynal A.S. (Oslo, Norway) and purchased from IGEN International, Inc. (Gaith-

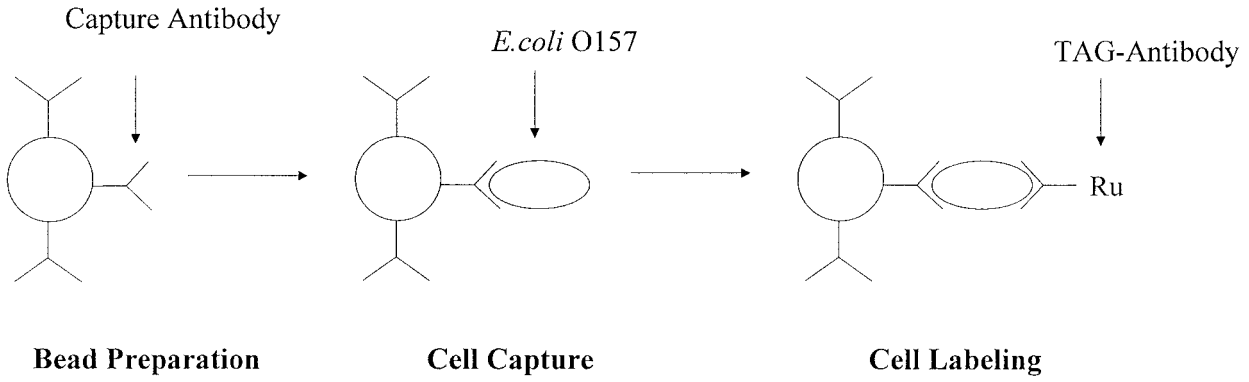

FIG. 1. Diagram of IM bead antibody-TAG sandwich assay.

ersburg, Md.). A monoclonal antibody to O157 LPS (1 mg) was obtained as a liquid suspension from BioDesign International (Kennebunk, Maine). The antibody was passed through a gel filtration column that was equilibrated with 150 mM PBS-1. To the monoclonal antibody solution in PBS-1 was added 75 μg of Biotin-LC-sulfoNHS ester (IGEN International, Inc.) from a stock solution of 2 mg ml^{-1} in water. After 1 h at ambient temperature the reaction was halted by the addition of 40 μmol of glycine (from a 2 M stock in water) followed by 10 min of incubation at room temperature to inactivate unreacted material. The biotinylated antibody was purified by passage through a gel filtration column equilibrated with 150 mM PBS-2. Biotinylated antibody was stored at 4°C.

An affinity-purified polyclonal antibody to *E. coli* O157 from a goat was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, Md.) as a freeze-dried preparation (1 mg). The antibody was dissolved in 0.3 M sodium phosphate buffer (pH 7.4) to a concentration of 10 mg ml^{-1} according to the manufacturer's instructions and then diluted to 1 mg ml^{-1} with PBS-1. To 1 mg of the antibody was added 56 μg of ORIGEN TAG-NHS ester [ruthenium (ii) tris-bipyridyl (referred to hereafter in this work as TAG) IGEN International, Inc.] from a stock solution of 1.5 mg ml^{-1} in dimethyl sulfoxide. The conditions for the reaction, termination of the reaction with glycine, purification of the antibody, and storage were identical to those described above for the biotinylation of antibodies. TAG-labeled antibodies were purified and stored in PBS-2 at 4°C. Protein (antibody) concentrations were determined using the bicinchoninic acid protein assay of Pierce Chemical Co. (Rockford, Ill.) with BSA as standard. Working antibody solutions were prepared by diluting with diluent.

IM-ECL protocol. The sandwich assay procedure is illustrated in Fig. 1. Preliminary experiments with PBS-2 and raw water samples were conducted with IM beads prepared immediately prior to use. Fifty microliters of strepavidin beads (0.4 mg ml^{-1}) was incubated for 30 min with 50 μl of biotinylated monoclonal antibody (1 μg ml^{-1}). To capture cells 0.5 ml of *E. coli* O157 cells was added to prepared IM beads and shaken for 2 h. To label cells 50 μl of the TAG antibody (1 μg ml^{-1}) was added, and the mixture shaken for an additional 2 h. (The final total volume was 650 μl. All water samples were run in triplicate. PBS-2 blanks and/or unspiked water samples (background) were run at the beginning and end of all standard curves.

For experiments with concentrated surface water samples, monoclonal IM beads were prepared in bulk and used as needed. Five milliliters of strepavidin beads (0.4 mg ml^{-1}) was incubated for 1 h with 5 ml of biotinylated monoclonal antibody (1 to 2 μg ml^{-1}). Beads were harvested using an MPC-1 magnetic particle collector (Dynal) and suspended in 1 ml of PBS-2, providing a 10-fold concentration. They were stored at 4°C until use. For cell capture, 20 μl of IM beads and 100 μl of diluent were added to 0.5 ml of water sample and shaken for 2 or 3 h. For sequential bead capture (SBC), 20 μl of IM beads and 100 μl of diluent were added to 1 to 3 ml of concentrated water sample and incubated for 3 h, the beads were recovered for 15 min using an MPC-S magnetic particle collector (Dynal), the supernatant was transferred to new microcentrifuge tubes, an additional 20 μl of IM beads, was added, and the tubes incubated for 3 h. Preliminary experiments indicated that a 15-min bead capture was adequate for quantitative recovery of beads from concentrated water samples (data not shown). To label cells, 50 μl of the TAG antibody (1 to 2 μg ml^{-1}) was added and the mixture was shaken for an additional 2 h. The final total volume was 650 μl. All water samples were run in triplicate or quadruplicate.

ECL instrumentation. Samples were analyzed using the ORIGEN device manufactured by IGEN International, Inc. Briefly, 550 μl of samples (85% of total volume) was pumped through a flow cell where the bead-cell-TAG complexes were magnetically captured on a platinum electrode, the sample was washed to remove contaminants and unused reagents, and a voltage was applied to create an electron transfer reaction in the presence of tripropylamine, resulting in the emission of multiple photons from the Ru-chelate component of TAG. The adjustable instrument parameters were an assay gain of 100, instrument background subtraction, and signal averaging. Approximate analysis time per tube was 75 s. Net ECL units are values for spiked water samples minus values for blank water samples.

Statistics. Detection limits for the sequential bead capture protocol were determined using Student's *t* test (23) comparing mean ECL signals from the first and second bead captures as follows: $t = d/s_{\bar{d}}$, where d is the difference between means, $S_{\bar{d}}$ is $(2s^2/n)^{1/2}$, s^2 is the sample variance, and n is the replicate number of tubes. Values of Student's *t* for 6 df are $P = 0.05$, 2.447; $P = 0.01$, 3.707; and $P = 0.001$, 5.959.

RESULTS

Protocol optimization with PBS and raw water. Three antibody sandwich formats were evaluated for quantitative detection of *E. coli* O157: commercial IM beads-polyclonal TAG, polyclonal IM beads-polyclonal TAG, and monoclonal IM beads-polyclonal TAG. Standard curves with commercial, polyclonal, and monoclonal IM beads all gave a dynamic range of ca. 10^1 to 10^5 cells ml^{-1} (data not shown). However, there were substantial differences in regression slopes and background ECL values. Average slopes ± standard deviations SDs) for commercial, polyclonal, and monoclonal IM beads were 0.83 ± 0.05 ($n = 4$), 0.68 ± 0.05 ($n = 4$), and 1.0 ± 0.03 ($n = 4$), respectively, while mean background values were 740 ± 130 ($n = 24$), 820 ± 230 ($n = 24$), and 164 ± 22 ($n = 36$) ECL units, respectively.

Monoclonal IM beads were tested with *E. coli* O157 strains Odwalla, B6914, and ATCC 35150. All strains gave comparable results (Fig. 2, upper panel). Monoclonal IM beads selectively captured *E. coli* O157 cells in the presence of ca. 10^6, 10^7, or 10^8 cells ml^{-1} of a sorbitol-negative non-O157 strain of *E. coli* (Fig. 2, lower panel). The dynamic range with monoclonal IM beads was from ca. 10^1 to 10^5 cells ml^{-1} (Fig. 3), although values for 10^5 cells ml^{-1} were somewhat variable, indicating that the maximum limit of linear detection was between 10^4 and 10^5 cells ml^{-1}. ECL signals at cell concentrations of $>10^5$ ml^{-1} decreased, presumably because cell concentrations exceeded IM bead or antibody-TAG concentrations.

Results obtained with spiked Little Cove Creek and Licking Creek water samples and monoclonal IM beads were similar to

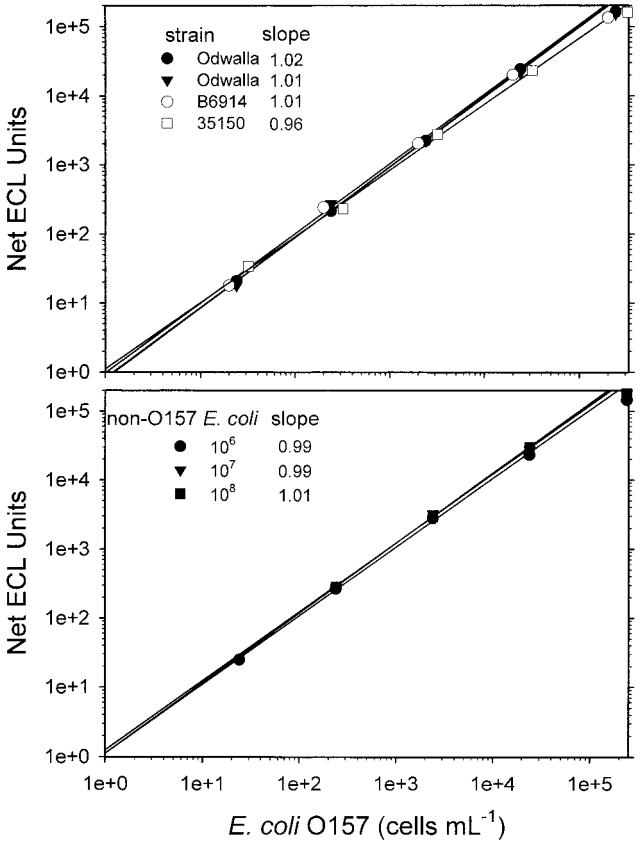

FIG. 2. The upper panel shows standard curves with 10^1 to 10^5 *E. coli* O157 strains Odwalla, B6914, and 35150 cells ml^{-1}, with monoclonal IM beads in PBS-2. The lower panel shows standard curves with 10^1 to 10^5 cells of *E. coli* O157 (Odwalla) per ml, using monoclonal IM beads in PBS-2 in the presence of 10^6, 10^7, or 10^8 non-O157 *E. coli*. Linear regression slopes were computed using 10^1 to 10^4 samples.

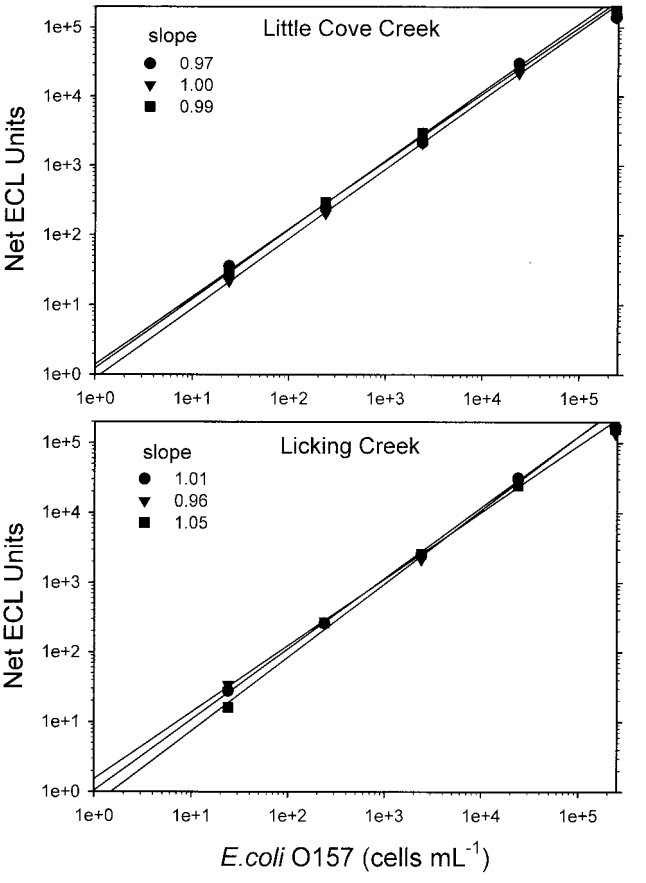

FIG. 4. Standard curves with 10^1 to 10^5 *E. coli* O157 (Odwalla) cells ml^{-1} in Little Cove Creek and Licking Creek water samples, with monoclonal IM beads; linear regression slopes were computed using 10^1 to 10^4 samples.

those obtained with PBS-2 (Fig. 4). Background ECL values for raw creek water samples were slightly higher than for PBS-2 blanks (cited above; Table 1). By comparison, background ECL values with commercial or polyclonal IM beads were substantially higher (Table 1). Filtering of water samples through a 0.2-μm-pore-size cellulose acetate filter had little effect on background values.

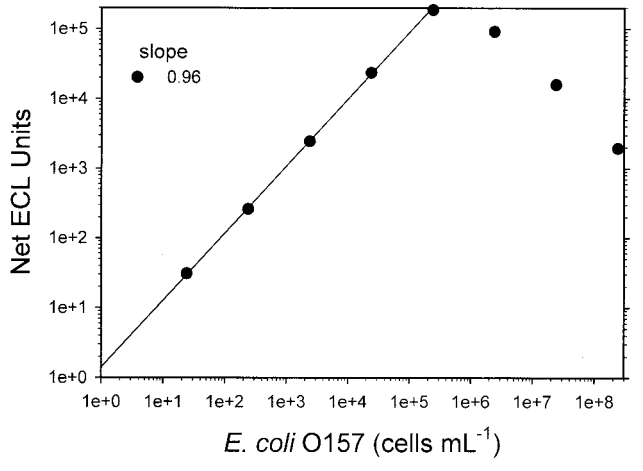

FIG. 3. Dynamic range of IM-ECL assay with 10^1 to 10^8 *E. coli* O157 (Odwalla)) cells ml^{-1} in PBS-2, with monoclonal IM beads; the linear regression slope was computed using 10^1 to 10^4 samples.

Protocol optimization with concentrated water. Preliminary studies with concentrated water samples indicated that background ECL signals were substantially higher than with PBS-2 or raw water, apparently due to nonspecific binding by unidentified materials in water samples. Experiments with concentrated water samples serially diluted with PBS-2 demonstrated that there was apparent saturation of binding sites (Table 2). Eight detergents or polymers were evaluated for their ability to

TABLE 1. Background ECL signals from unfiltered and filtered (0.2-μm pore size) Little Cove Creek and Licking Creek water samples

IM beads	Background ECL units (mean ± SD) in water[a] from:			
	Little Cove Creek		Licking Creek	
	Unfiltered	Filtered	Unfiltered	Filtered
Commercial	4,577 ± 237	4,367 ± 265	8,079 ± 422	8,141 ± 685
Polyclonal	2,070 ± 224	1,720 ± 177	2,956 ± 372	3,027 ± 292
Monoclonal	171 ± 14	179 ± 35	174 ± 21	178 ± 18

[a] $n = 12$.

TABLE 2. Background ECL signals from concentrated Little Cove Creek and Licking Creek water samples diluted with PBS-2

Concn of water(%)	Background ECL units in water from:	
	Little Cove Creek	Licking Creek
0	124	122
20	458	270
40	587	435
60	776	463
80	740	495
100	748	527

minimize background nonspecific binding: glycerol, Triton X-100, PEG 1000, PEG 8000, BSA, PVPP, hexa meta-phosphate, and EDTA. Only PVPP at 0.1% reduced background values to near those of PBS-2 (data not shown). However, the reaction of 0.1% PVPP with cell cleaning solution routinely clogged the ORIGEN inlet tubing. Experiments with lower concentrations indicated that the maximum concentration which would not plug the tubing was 0.005%. PVPP reduced background ECL signals ca. 50% in 2-h incubations and ca. 30% in 3-h incubations (data not shown). Limited studies were conducted to assess the nature of the nonspecific binding. When concentrated water samples were incubated with antibody-conjugated beads or nonconjugated beads (2 h), background ECL values (with 0.005% PVPP) were higher for nonconjugated beads (646 ± 98) than for antibody-conjugated beads (349 ± 42; $n = 6$).

Standard curves were run with Little Cove Creek and Licking Creek concentrated (100-fold) water; turbidity was 400 to 500 NTU. ECL signals were somewhat diminished relative to PBS-2 or raw water standard curves (Fig. 5). The combination of higher backgrounds and diminished ECL signal per cell resulted in lower net ECL signals. Consequently, the sensitivity of the assay was reduced to ca. 10^{-2} *E. coli* O157 cells ml of concentrated water^{-1} (Fig. 5). However, neither turbidity nor PVPP adversely effected the linearity or dynamic range.

The ability of consecutive cell capture incubations, termed

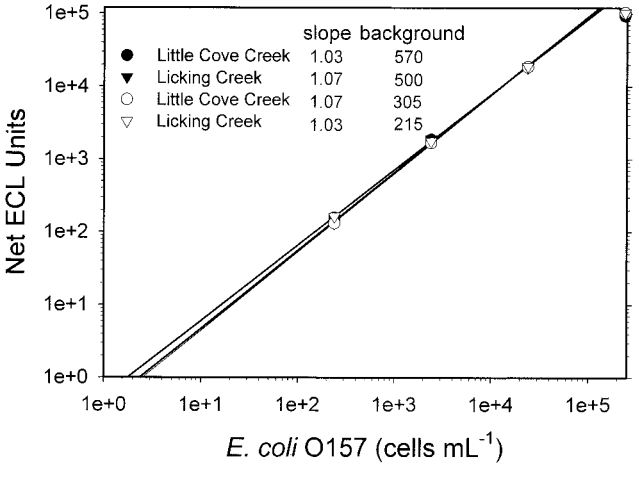

FIG. 5. Standard curves for *E. coli* O157 (Odwalla) cells in concentrated (100-fold) Little Cove Creek and Licking Creek water samples with (closed symbols) or without (open symbols) 0.005% PVPP; linear regression slopes were computed using 10^1 to 10^4 samples.

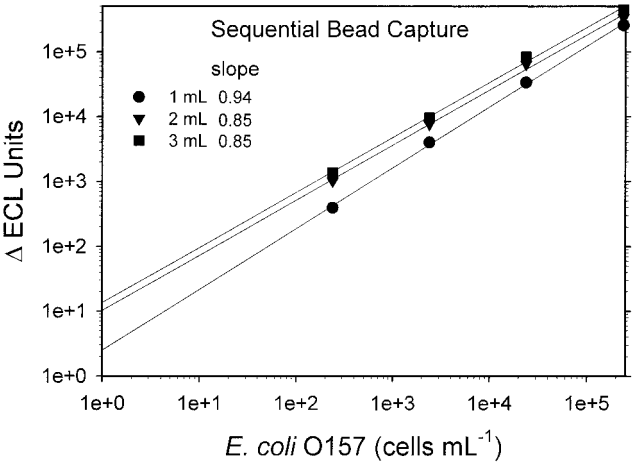

FIG. 6. Standard curves for *E. coli* O157 (Odwalla) cells in concentrated (100-fold) and centrifuged (100 × *g*) Little Cove Creek water samples, obtained after SBC with 1-, 2-, and 3-ml sample volumes; combined net ECL signals from the first and second bead captures are shown for a cell concentration of 10^5.

SBC, to establish baseline values for individual water samples was investigated. These experiments were conducted with a new preparation of polyclonal TAG-antibody which gave a higher and somewhat more variable ECL signal. For all experiments, monoclonal IM beads were prepared in advance as previously described. PVPP 0.1% was added to stock bead preparations, providing a final PVPP concentration in tubes of ca. 0.0033%.

Based on ECL signals, ca. 80% of *E. coli* O157 cells were captured in the initial cell capture incubation (2 h), with the remainder being captured in the successive incubation (data not shown). Extending the cell capture incubation to 3 h resulted in enhanced cell recoveries of >90% (data not shown). At cell concentrations of $\leq 10^3$ cells ml^{-1}, ECL values for the consecutive cell capture (second bead capture) were comparable to background values; i.e, the differences in ECL signal between the first and second bead captures (ΔECL) were consistent with net ECL responses from standard curves. At cell concentrations of $>10^4$ cells ml^{-1}, however, ECL values for the consecutive cell capture (second bead capture) were substantially higher than background values due to the ECL signal from cells captured during the consecutive cell capture. Since the typical background levels observed for concentrated waters were ≤1,000 ECL units, a more accurate estimate of cell concentrations of $>10^4$ ml^{-1} was obtained by combining ECL signals from the first and second bead captures. The SBC assay gave a linear dynamic range of ca. 10^2 to 10^5 cells ml^{-1} in concentrated water (Fig. 6). ΔECL values were correlated with the sample volume; increasing sample size from 1 to 3 ml resulted in an ~2.5-fold enhancement in sensitivity (Fig. 6).

Protocol validation with concentrated water. Triplicate 10-liter Little Cove Creek water samples were spiked with ca. 5,000 *E. coli* O157 cells and concentrated, and 1- and 3-ml aliquots were analyzed by SBC (Fig. 7; Table 3). Concentration via vortex filtration did not appear to affect cell viability; total coliform recoveries on agar plates (including *E. coli* O157) from concentrate were consistent with initial populations of

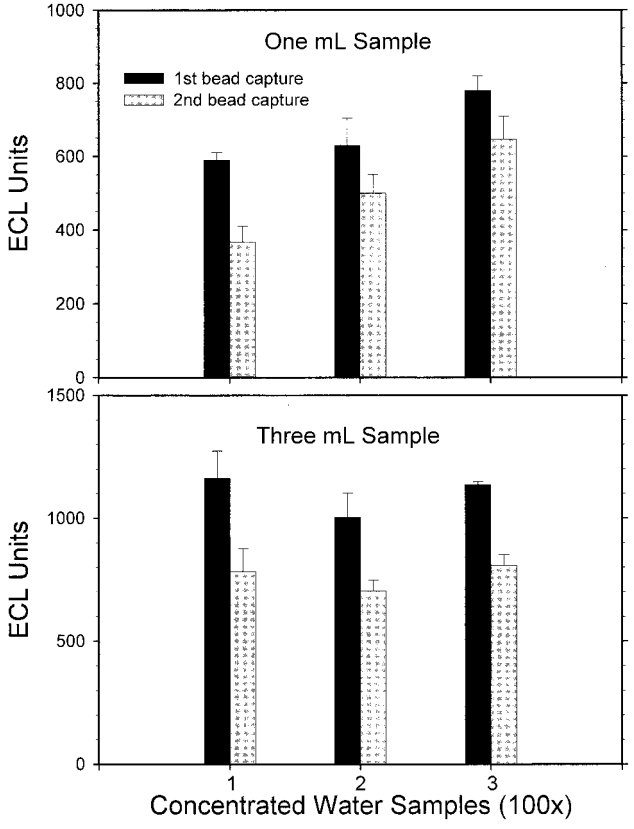

FIG. 7. Mean ECL signals and SDs (error bars) ($n = 4$) for ca. 5,000 *E. coli* O157 (Odwalla) cells spiked into 10 liters of Little Cove Creek water after concentration (100-fold) and centrifugation ($100 \times g$).

spiked plus background coliforms (Table 3). In the absence of low-speed centrifugation ($100 \times g$), there was no difference in ECL signals between the first and second bead captures (data not shown); i.e., *E. coli* O157 cells were not detected. Low-speed centrifugation decreased NTU values ca. 10-fold; however, based on plating before and after centrifugation, low-speed centrifugation did not result in a detectable loss of viable bacteria (data not shown). Increasing the sample volume from 1 to 3 ml increased the ΔECL values more than twofold, consistent with previous experiments (Fig. 7; Table 3). The

TABLE 3. Recoveries of total coliforms from concentrated water samples and ΔECL values from SBC[a]

Water sample[b]	NTU[c]	Cells recovered ml^{-1}	ΔECL (t) from SBC	
			1 ml	3 ml
1	35	54[d]	221 (13.0)[g]	379 (7.4)[g]
2	46	54[e]	130 (3.1)[g]	300 (7.9)[g]
3	43	63[f]	133 (5.1)[g]	328 (14.6)[g]

[a] Total coliforms (lactose positive), including *E. coli* O157, from concentrated (100-fold) water samples. ΔECL values are from 1- and 3-ml SBC, and statistical significance was determined by using Student's t test.
[b] Water samples (10 liters) spiked with ca. 5,000 *E. coli* O157.
[c] NTU reading after centrifugation ($100 \times g$).
[d] Includes seven total coliforms 100 ml^{-1} in original water sample.
[e] Includes eight coliforms 100 ml^{-1} in original water sample.
[f] Includes nine total coliforms 100 ml^{-1} in original water sample.
[g] Highly statistically significant ($P < 0.01$).

TABLE 4. Enrichment tubes positive for the presence of *E. coli* O157 (>10,000 ECL units)

Sample	No. of tubes positive for O157/total no. of tubes	
	ECB[a]	MLB[b]
Unspiked water	0/9	0/9
Spiked water	1/3, 2/3[c]	2/3, 3/3[c]
Concentrated water (100-fold)	9/9	9/9

[a] All tubes were turbid regardless of the presence of *E. coli* O157.
[b] Turbidity was directly correlated with presence of *E. coli* O157.
[c] Number of positive tubes for water samples 2 and 3; the positivity for sample 1 was not determined.

ΔECL values from two of three 1-ml samples were highly statistically significant ($P < 0.01$), while all three values from 3-ml samples were highly statistically significant ($P < 0.01$) (Table 3).

Experiments were conducted to detect viable *E. coli* O157 cells via enrichment culture. With spiked raw water samples, a higher percentage of tubes were positive for *E. coli* O157 with MLB than ECB (>10,000 ECL units) (Table 4). With concentrated water samples, all tubes (both MLB and ECB) were positive for *E. coli* O157 (Table 4). MLB was more selective than ECB. All ECB tubes became turbid, including unspiked, spiked, and concentrated water samples, while only those MLB tubes which were positive for *E. coli* O157 became turbid.

The effect of bile salts on ECL response was investigated. Net ECL signals (normalized to 10^2, 10^3, or 10^4 cells ml^{-1}) were highest for stock culture cells (grown in L broth), while net ECL signals for MLB cells grown with bile salts were ca. three-fold higher than those for cells grown without bile salts (Fig. 8).

DISCUSSION

Although commercial, polyclonal, and monoclonal anti-O157:H7 IM beads gave comparable dynamic ranges of ca. 10^1 to 10^5 cells ml^{-1} in PBS-2, the monoclonal IM beads were chosen for further development and optimization based on background ECL levels, selectivity, and linearity. Background

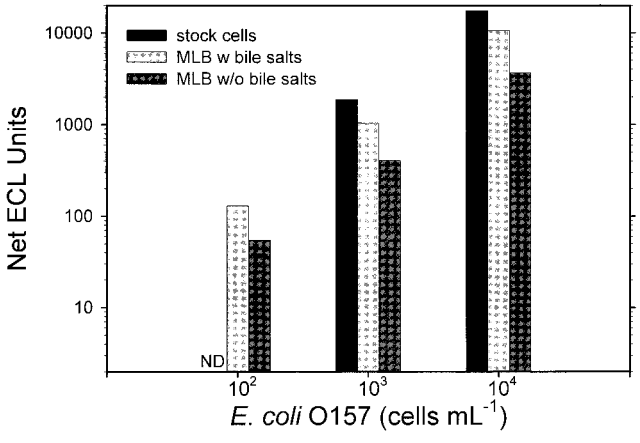

FIG. 8. Net ECL signal (normalized) for *E. coli* O157 (Odwalla) cells in stock culture or cultured in 1 mM MLB with or without bile salts (mean of three cultures). ND, not determined.

ECL values with commercial and polyclonal IM beads for raw creek water samples ranged from ca. 2,000 to 8,000, effectively masking *E. coli* O157 cell concentrations of $\leq$100 cells ml^{-1}. Similar ECL values were obtained with filtered and unfiltered surface water samples, suggesting that high background ECL values were not due to *E. coli* O157 but rather to nonspecific binding to beads by unidentified organic materials. Background ECL values with monoclonal IM beads were only slightly higher in surface water samples than in PBS-2. This suggests that for low turbidity samples (NTU $<$ 5) it may be feasible to use PBS-2 values as baseline values.

An anti-O157 LPS monoclonal antibody was chosen for immunomagnetic capture to maximize selectivity. Other investigators have reported cross-reactivity between commercial polyclonal *E. coli* O157 IM beads and non-O157 strains (16). Using monoclonal IM beads, small numbers of *E. coli* O157 cells were detected and quantified in the presence of large numbers of a non-O157 strain, strain 794. The 794 strain was isolated as a presumptive *E. coli* O157 because it was sorbitol negative. Based on information from BioDesign, the monoclonal immunoglobulin G O157 antibody does not react with related *E. coli* strains. However, cross-reactivity with other bacteria is unknown and should be evaluated.

An anti-O157 polyclonal antibody was chosen as the TAG-antibody based on the hypothesis that this would maximize TAG binding sites, hence ECL signal. The combination of monoclonal IM beads and polyclonal TAG-antibody consistently gave linear slopes of ca. 1.0 in PBS-2 and raw water (from $>10^1$ to $\leq 10^5$ cells ml^{-1}), indicative of a constant ECL response per cell. Consequently, analyzing a single cell concentration should be adequate to establish a standard response curve.

Low ECL background levels in low-turbidity raw water samples (relative to PBS-2 blanks) allowed for rapid screening. However, assay sensitivity (ca. 25 cells ml^{-1} or 2,500 cells 100 ml^{-1}) was inadequate to detect *E. coli* O157 at levels which could present a public health threat. Preliminary experiments with concentrated water samples (vortex filtration) indicated that background levels were substantially elevated over PBS-2 blanks due to nonspecific binding. Experiments with nonconjugated IM beads suggested that unidentified materials were binding directly to the bead matrix or streptavidin sites, as opposed to the monoclonal antibody. PVPP partially inhibited this nonspecific binding. PVPP was added directly to stock IM bead preparations (0.1%), resulting in a final concentration of ca. 0.0033% in incubation tubes. At this concentration, background ECL signals were reduced ca. 50% during a 2-h incubation or ca. 30% during a 3-h incubation. Although helpful, PVPP is only a partial remedy. A more ideal solution would be to identify and permanently block the IM bead binding sites to prevent nonspecific binding to the bead matrix or streptavidin sites.

Attempts to establish baseline values for individual water samples using filtration were unsuccessful (data not shown). Substantial numbers of cells from the stock culture passed through the 0.2-μm-pore-size filter. Although these results are not typical for viable *E. coli* cells, they do suggest that filtration could provide incorrect baseline data if small cells or cell fragments are present in water samples.

The SBC method was developed to accurately account for background ECL signals. The SBC method is based on the premise that the ECL signal from the initial cell capture incubation is due to cell capture plus background binding while the second incubation is due predominately to background binding. This assumes that background ECL signals are consistent between the first and second bead captures. Our studies indicated that the material(s) in Little Cove Creek and Licking Creek concentrated water samples responsible for the background signal were present in excess. As a consequence, background ECL signals from the first and second bead captures were consistent. Further research is required to determine if this is universally true.

Experiments were conducted to evaluate and optimize the SBC protocol. A 2-h incubation resulted in ca. 80% cell capture. Increasing the incubation time to 3 h improved recoveries to $>$90%. At cell concentrations of $>10^4$ ml^{-1} (ca. 20,000 ECL units), the ECL signal from the second bead capture was higher than the background signal, due apparently to incomplete capture. At these high cell concentrations, the addition of ECL signals from the first and second bead captures provided a more accurate estimate of *E. coli* O157 populations. Increasing the sample volume from 1 to 3 ml increased sensitivity ca. 2.5-fold. The protocol consistently provided linear data over the effective dynamic range (ca. 10^2 to 10^5), indicative of a constant ECL signal per cell.

Spiking experiments were conducted to validate the protocol and estimate detection limits. Vortex filtration provided essentially complete recoveries of *E. coli* O157 cells spiked into water samples. The SBC protocol successfully detected *E. coli* O157 (Odwalla) cells in the spiked water samples, and the ΔECL values obtained were generally consistent with standard curves. Utilizing regression data for the 3-ml volume standard curve shown in Fig. 6 resulted in an estimated cell concentration of 60 cells ml^{-1}, after correcting for growth medium effects (see below), which was comparable to the expected value of 50 cells ml^{-1}. The maximum sensitivity of the assay is dependent on the magnitude of the difference in ECL signals between first and second bead captures (ΔECL), variability within replicates, and the number of replicates. We chose four replicates as a compromise between cost, labor, and statistical power. Student's *t* test for paired comparisons was used to determine assay sensitivity. These data suggest that with a 3-ml incubation the detection limit could be as low as 25 *E. coli* O157 cells 100 ml^{-1} ($P < 0.01$), assuming comparable variability for other water samples.

Enrichment cultures with raw spiked samples suggest that both ECB and MLB are potentially capable of enriching one viable cell. Consequently, the combination of concentration, enrichment, and IM-ECL analysis can potentially achieve a detection limit of 1 to 2 viable cells liter^{-1}, depending on the volume of water concentrated ($\geq$10 liters) and the volume of concentrated sample used for enrichment. MLB appeared to be more selective than ECB, because it selected only for organisms capable of growth with lactose as the sole carbon source. In the absence of spiking, no MLB tubes were turbid. Further research is necessary to determine the rates of false negatives with both ECL and MLB. False negatives may arise with ECB if *E. coli* O157 cells are out-competed by organisms growing on casein, while false negatives may arise with MLB if *E. coli* O157 cells are unable to adapt to the minimal medium.

The use of monoclonal IM beads should minimize false positives, although this also requires further investigation.

Standard curves with stock culture Odwalla cells versus MLB-grown cells, with and without bile salts, indicate that it may be impossible to establish a definitive standard curve for *E. coli* O157. The ECL signal per cell was highest with the stock culture. It is plausible that the original hemocytometer counts for the stock culture were underestimated. However, note that stock cells were of a diminished size, as evidenced by the fact that a majority of cells passed through a 0.2-μm-pore-size filter. Since the ECL photochemical reaction only occurs within close proximity to the electrode surface, cell geometry (i.e., total cell surface area within the reaction zone) directly affects the magnitude of the ECL signal. The differences between MLB with and without bile salts are not attributable to incorrect cell numbers, because cells were cultured simultaneously on similar media and gave essentially identical CFU and hemocytometer counts. These data suggest that *E. coli* O157 (Odwalla) cells modulate their outer cell membrane composition in response to bile salts. Consequently, the IM-ECL signal is directly dependent upon the growth culture media, both in vivo and in vitro.

The IM-ECL assay appears suitable for routine analysis and screening of water samples. Several similar immunological methods have been described with varying sensitivities, analysis times, and levels of sophistication. It is difficult to compare the IM-ECL protocol to these methods, however, because of limited information regarding their applicability or optimization for raw or concentrated surface water samples. Since the IM-ECL assay detects nonviable, viable but nonculturable, and culturable cells, it may be useful in locating and "tracking" *E. coli* O157 plumes or identifying point sources after waterborne infections have occurred. In conjunction with enrichment procedures, the protocol can also be used to detect viable cells. The generic protocol is potentially applicable to a wide range of microorganisms (bacteria, protozoa, and viruses). The application of the assay to other specific pathogens, however, will be dependent on the availability of appropriate antibodies with the selectivity to allow capture and labeling of the target population.

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